Mutations in the "lid" region affect chain length specificity and thermostability of a *Pseudomonas fragi* lipase

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Abstract The cold-adapted *Pseudomonas fragi* lipase (PFL) displays highest activity on short-chain triglyceride substrates and is rapidly inactivated at moderate temperature. Sequence and structure comparison with homologous lipases endowed with different substrate specificity and stability, pointed to three polar residues in the lid region, that were replaced with the amino acids conserved at equivalent positions in the reference lipases. Substitutions at residues T137 and T138 modified the lipase chain-length preference profile, increasing the relative activity towards C8 substrates. Moreover, mutations conferred to PFL higher temperature stability. On the other hand, replacement of the serine at position 141 by glycine destabilized the protein.

Keywords: Lipase; Chain length selectivity; Thermostability; *Pseudomonas fragi*

1. Introduction

Lipases catalyze the hydrolysis of triacylglycerols and several other substrates containing ester bonds [1,2] with an astonishing variety in their regio- and stereo-specificity as well as in their selectivity towards the length and saturation degree of the acyl chain. Rational and random mutagenesis studies have been performed to the end of modifying their catalytic performances [3]. In particular, chain length specificity has been addressed in fungal and bacterial lipases, among others the lipases from *Candida rugosa* [4,5], *Geothricum candidum* [6], *Rhizopus delemar* [7] *Humicola lanuginosa* [8] and *Burkholderia cepacia* [9]. Rational mutagenesis can build on the observation that, despite a marked divergence in primary sequence, all lipases conform to a common structural organization, named the alpha/beta hydrolases fold [10]. Access to the active site – a serine, histidine, carboxylic acid triad – may be shielded by a mobile lid, whose position – closed or open – determines the enzyme in an inactive or active conformation. The substrate-binding site is located inside a pocket on top of the central β-sheet typical of this fold. Size and geometry of the substrate-binding cleft have been related to substrate specificity [11] and residues that contact the substrate have been identified by crystallography and docking, making this part of the structure a major target for mutagenesis. However, it is recognized that other protein regions, such as the lid itself [4,12,13] and the reaction conditions [14], may play a role in lipase's specificity.

We have recently described the catalytic properties of the lipase from *Pseudomonas fragi* (PFL, [15]), an enzyme endowed with activity at low temperature and a marked selectivity towards short-chain substrates, and built a model of its 3D structure by homology with the *B. cepacia* (BCL, [16]), *Pseudomonas aeruginosa* (PAL, [17]) and *Burkholderia glumae* (BGL, [18]) lipases, to which it shares high sequence identity. Despite their close relatedness in sequence and structure, these enzymes differ in their profiles for chain length specifity, with PFL active on short-chain triglycerides [15], PAL and BGL with a broad substrate specificity [19] and BCL with a high preference for the hydrolysis of triglycerides with a chain length $\geqslant 8$ [20]. Moreover, the three homologous lipases are more thermostable. The enzyme–substrate interaction has been studied in detail for PAL and BCL crystallized in complex with the same triglyceride-like inhibitor [17,21] allowing for the identification and mutagenesis of residues relevant for substrate recognition [9,17,22].

In this study, we focused on residues of the lid region to explore its role in substrate recognition. Interestingly, substitution with the corresponding amino acids of homologous lipases affected not only selectivity but also the temperature stability of the thermolabile *P. fragi* enzyme.

2. Materials and methods

2.1. PCR mutagenesis

Mutagenesis was performed on the gene encoding the lipase from *P. fragi* strain IFO3458 cloned in plasmid pQE30 (Quiagen) fused at its N-terminus with a His_6 tag for purification [15]. As the cloning host *Escherichia coli* JM101 (Promega Co, WI, USA) was used. Heterologous expression was performed in the *E. coli* strain SG13009[pREP4] (Qiagen). Site-directed mutagenesis was carried out using the kit QuickChange™ (Stratagene, CA, USA) and mutagenic oligonucleotide primers. Primer sequences were as follows:
T137V forward: 5′-GTGGCTGCCGCCCTGgtCACCTCGTTC-3′;
T137V reverse: 5′-GAACGAGGTGacCAGGGCGGCAGCCAC-3′;
T138N forward: 5′-TGACgAatTCGTTCAGCGCATTTTTATCCG-3′;
T138N reverse: 5′-ACGAatTcGTCAGGGCGGCAGCCACCG-3′;

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Abbreviations: PFL, *Pseudomonas fragi* lipase; BCL, *Burkholderia cepacia* lipase; BGL, *Burkholderia glumae* lipase; PAL, *Pseudomonas aeruginosa* lipase; wt, wild-type; PCR, polymerase chain reaction; dNTP, deoxynucleotide triphosphate

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S141G forward: 5′-ACCACtagtTTCgGCGCATTTTTATCCGCC-3′; S141G reverse: 5′-GCGCcGAAactaGTGGTCAGGGCGGCAG-3′. Polymerase chain reaction (PCR) was performed in a final volume of 20 μl containing 20 ng template DNA, 0.2 mM deoxynucleotide triphosphates (dNTPs), 1 μM oligonucleotide primers and 0.5 U of PfuTurbo™ polymerase (Stratagene, CA, USA). The amplification program was as follows: 3 min at 95 °C, followed by 14 cycles of 30 s at 95 °C, 60 s at 55 °C and 8 min at 68 °C. A final digestion step with *Dpn*I removed template DNA. Other DNA manipulations were according to [23] and manufacturer's instructions for the enzymes and materials employed.

2.2. Expression and purification

E. coli SG13009 transformed with the expression plasmid was grown overnight at 27 °C in Luria–Bertani medium supplemented with 100 μg/ml ampicillin and 25 μg/ml kanamycin. 0.4 mM Isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added and cultivation was continued for 4 h. Lipase was extracted and purified as described [15]. The protein concentration was determined according to [24] with bovine serum albumin used as the standard.

2.3. Activity assays

Lipase activity was determined in a pH-stat assay by titrating fatty acids released from triacylglycerols with 0.01 M sodium hydroxide using a 718 STAT TITRINO (Metrhom). Emulsions of 20 mM triacylglycerols with 2% arabic gum were used as the substrate. Substrate specificity was determined at 29 °C, pH 8.0, on triacylglycerol substrates with growing chain lengths, i.e., tributyrin (C4), tricaprylin (C8), trilaurin (C12). The effect of temperature on enzyme activity was determined by preincubating protein samples at 29 °C before determining residual activity on tricaprylin as above.

2.4. Structure inspection

The three-dimensional structures of BCL, PAL and BGL were obtained from the Protein Data Bank (PDB codes: 2LIP, 1EX9 and 1CVL, respectively) whereas the structure of PFL, obtained by homology modeling, was taken from [15]. Protein structures have been visualized using the software package VMD [25].

3. Results

3.1. Selection of amino acid residues for substitution

Fig. 1 shows a structure-based alignment of the PFL sequence with three highly homologous lipases of known 3D structure. The four proteins share 35–43% identity in their primary sequence yet markedly differ in chain length preference and thermostability [19,20]. Features unique to PFL are located in three functional regions: the lid loop (residues 128–148), the calcium-binding site (concerning residues H266 and R269) and the substrate-binding site [15]. Aiming to clarify whether the lid may be considered as an important functional determinant, as in other lipases [4,12,13], we specifically targeted this part of the protein. Sequence inspection allowed to identify residues T137, T138 and S141 that substitute amino acids strictly conserved in BCL (V143, N144, G147), PAL (V135, N136, G139) and BGL (V143, N144, G147) with polar residues (Fig. 2). In fact, hydrophobicity of the substrate-binding site together with its size and shape has been shown

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BCL    ADNYAATRYPIILVHGLTGTDKYAGVLEYWYGIQEDLQQRGATVYVANLSGFQSDDGPNG 60
BGL    ADTYAATRYPVILVHGLAGTDKFANVVDYWYGIQSDLQSHGAKVYVANLSGFQSDDGPNG 60
PAL    -STYTQTKYPIVLAHGMLGFDNILG-VDYWFGIPSALRRDGAQVYVTEVSQLDTSE---V 55
PFL    MDDSVNTRYPILLVHGLFGFDRIGS-HHYFHGIKQALNECGASVFVPIISAANDNE---A 56

                                  *
BCL    RGEQLLAYVKTVLAATGATKVNLVGHSQGGLTSRYVAAVAPDLVASVTTIGTPHRGSEFA 120
BGL    RGEQLLAYVKQVLAATGATKVNLIGHSQGGLTSRYVAAVAPQLVASVTTIGTPHRGSEFA 120
PAL    RGEQLLQQVEEIVALSGQPKVNLIGHSHGGPTIRYVAAVRPDLIASATSVGAPHKGSDTA 115
PFL    RGDQLLKQIHNLRRQVGAQRVNLIGHSQGALTARYVAAIAPELIASVTSVSGPNHGSELA 116


BCL    DFVQGVLAYDPTGLSSTVIAAFVNVFGILTS--SSNNTN-QDALAALKTLTTAQAATYNQ 177
BGL    DFVQDVLKTDPTGLSSTVIAAFVNVFGTLVS--SSHNTD-QDALAALRTLTTAQTATYNR 177
PAL    DFLR---QIPPGSAGEAVLSGLVNSLGALISFLSSGSTGTQNSLGSLESLNSEGAARFNA 172
PFL    DRLRL--AFVPGRLGETVAAALTTSFSAFLSALSGHPRLPQNALNALNALTTDGVAAFNR 174


BCL    NYPSAGLGAPGSCQTGAPTETVGGNTHLLYSWAGTAIQPTISVFGVTGATDTSTIPLVDP 237
BGL    NFPSAGLGAPGSCQTGAATETVGGSQHLLYSWGGTAIQPTSTVLGVTGATDTSTG-TLDV 236
PAL    KYP---QGIP-TSACGEGAYKVNGVSY--YSWS----GS------------------SPL 204
PFL    QYP---QGLP-DRWGGMGPAQVNAVHY--YSWSGIIKGSRL----------------AES 212


                                  *                   *
BCL    ANALDPSTLALFGTGTVMVNRGSGQNDGVVSKCSALYGQVLSTSYKWNHLDEINQLLGVR 297
BGL    ANVTDPSTLALLATGAVMINRASGQNDGLVSRCSSLFGQVISTSYHWNHLDEINQLLGVR 296
PAL    TNFLDPS-DAFLGASSLTFKNGT-ANDGLVGTCSSHLGMVIRDNYRMNHLDEVNQVFGLT 262
PFL    LNLLDP-LHNALRVFDSFFTRETRENDGMVGRFSSHLGQVIRSDYPLDHLDTINHMARG- 270


BCL    GANAEDPVAVIRTHANRLKLAGV 320
BGL    GANAEDPVAVIRTHVNRLKLQGV 319
PAL    SLFETSPVSVYRQHANRLKNASL 285
PFL    SRRRINPVELYIEHAKRLKEAGL 293
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Fig. 1. Alignment of amino acid sequences of BCL (P22088), BGL (Q05489), PAL (P26876) and PFL (AJ250176). The lid region is boxed and positions targeted by mutagenesis are in bold. Stars mark the catalytic residues and residues involved in calcium binding are boxed and shadowed. For the sake of clarity residues participating in substrate binding are not reported, as they are spread over the sequence (detail in [15]).

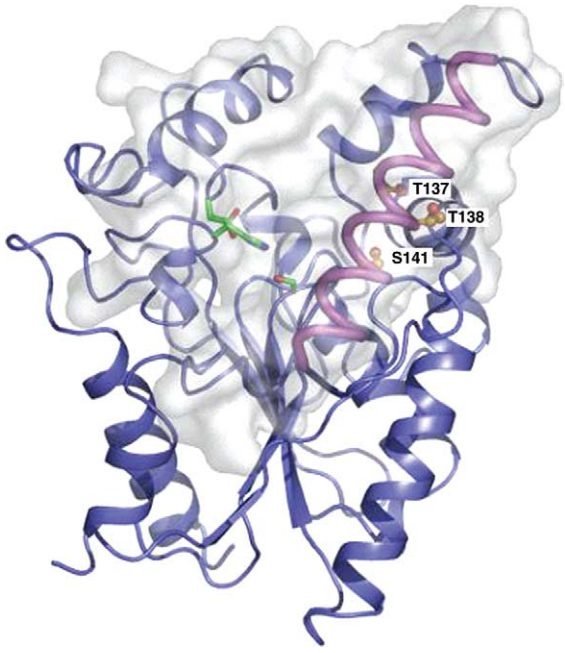

Fig. 2. 3D model of the PFL protein showing the lid in violet and the position of residues mutated. PFL active site is marked by the side chains of catalytic residues and the entrance of the substrate-binding tunnel enclosing the inner face of the lid is evidenced through the surface representation.

to affect chain length selectivity [11]. Moreover, polar residues in the surface-exposed lid might be involved in interactions with the solvent and, as a consequence, in protein flexibility.

3.2. Chain length preference profile of mutated lipases

Selected residues were therefore substituted with the amino acids conserved in the reference lipases. Single mutants on each position and combinations thereof were expressed in *E. coli* and purified by metal chelating chromatography as described previously [15]. In a standard activity assay performed on tributyrin at 29 °C, specific activity was 180 U/mg for the wild-type (wt) protein, 100 U/mg for the single mutants and 45 U/mg for the multiple mutants. Reduced activity is often observed in mutant proteins. To take this observation in due account, the relative hydrolysis rate of the lipases towards triglycerides of growing chain length was determined to reveal the shift of substrate specificity. Determined selectivity profiles are shown in Table 1, where activity on the C4 substrate is taken as 100%. The activity of wt PFL and of the mutants decreased with chain length. However, both substitutions on the lid positions 137 and 138 were found to increase the relative activity toward C8, whereas introduction of a glycine residue at position 141 affected selectivity towards trilaurin with respect to the wt protein. Multiple substitutions did not produce obvious additive effects.

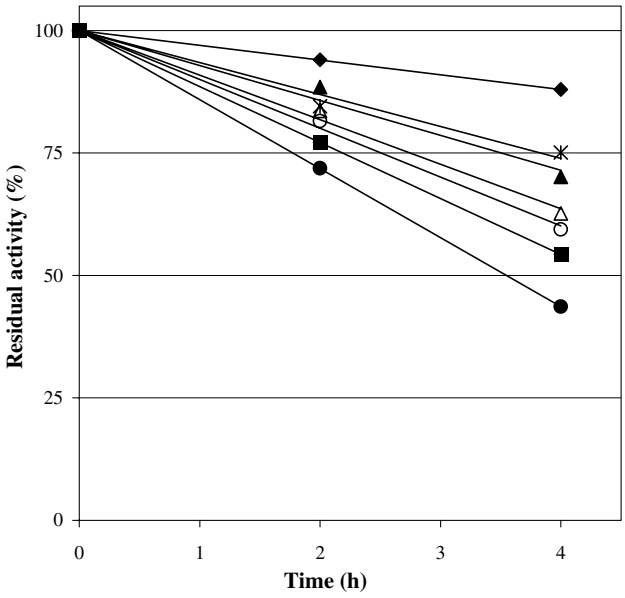

Fig. 3. Thermal inactivation of lipase proteins at 29 °C. ■: wt; ♦: T137V; ▲: T138N; ●: S141G; △: T137V–T138N; ○: T137V–S141G; ∗: T137V–T138N–S141G. Residual activities were calculated from the data of three experiments. The S.D. was <5%.

3.3. Effect of mutations on PFL temperature stability

The bacterial lipases considered in this study differ from each other, besides in substrate specificity, also in their temperature-dependent behavior. PFL, in fact, can be considered as a cold-adapted enzyme, as it is stable over a long time at 10 °C, whereas it displays a half life of 5 h at 27 °C and only of a few minutes at 50 °C. On the contrary, BCL is thermostable, PAL and BGL are mesophilic enzymes. Therefore, it was of interest to assess whether the above substitutions affect temperature stability. Based on previous data on the temperature dependence of the enzyme [15], we decided to follow the time-dependent loss of activity at 29 °C, a temperature where subtle changes in stability can be easily monitored. As shown in Fig. 3, activity of the wt lipase on tricaprylin was reduced by 50% after 4 h incubation, whereas, under the same conditions, mutant T137V still retained 90% of its activity and mutant T138N 70%. On the contrary, substitution S141G was found to destabilize the protein. As for substrate selectivity, even the effect of mutations on the temperature stability of PFL seems to be not additive.

4. Discussion

We have approached the issue of acyl chain length selectivity and protein stability starting from a comparison of bacterial lipases tightly related in sequence but endowed with different properties. Replacement of T137, T138 and S141 in the PFL

Table 1
Relative hydrolysis rates of wt and mutant *P. fragi* lipases on tricaprylin (C8) and trilaurin (C12)/tributyrin (C4)

	WT	T137V	T138N	S141G	T137V/T138N	T137V/S141G	T137V/T138N/S141G
C8/C4	0.41	0.60	0.67	0.47	0.60	0.66	0.68
C12/C4	0.16	0.18	0.09	0.29	0.11	0.19	0.10

The relative hydrolysis rates were calculated from the data of three experiments. The S.D. was <8%.

lid substitute a polar residue with the hydrophobic or basic side chains conserved in homologous lipases. Mutations T137V and T138N affected the preference for C8 triglycerides. One might hypothesize that improved performances on medium-chain substrates are mediated by direct interaction of the modified lid with fatty acid chains. Unfortunately, structural data of the enzyme in complex with substrate molecules are not available, making any hypothesis about the specific role of substitutions rather speculative. Thus, basing on the homology model in our hands, side chains of residues 137 and 138 should point to different directions. On the other hand, molecular dynamics calculations (not shown) suggest a high flexibility of the central region of the lid, where residues 137 and 138 are embedded. In this hypothesis, as time evolves the two side chains might assume alternately the same orientations, which would explain their overlapping effect. Consistent observations on the importance of the hydrophobicity of the substrate-binding site have been reported for mutants of *R. delemar* lipase, where introduction of polar residues resulted in an improvement of activity toward tricaprylin in comparison to triolein [26]. However, at the present stage of our knowledge, we cannot exclude that mutations in the lid might affect the enzyme conformation rather than being directly related to a specific enzyme–substrate interaction, as the existence of lipase conformers with the lid in intermediate positions has been shown to affect chain length preference [27]. It is noteworthy that two site specific substitutions with residues conserved in more stable enzymes contributes higher stability to a very labile protein. On the other hand, the destabilizing effect of substitution S141G might be related to the high flexibility of the glycine residue. Saturation mutagenesis experiments on this position may help in clarifying whether increased instability is specifically related to the presence of glycine.

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References

[1] Schmid, R.D. and Verger, R. (1998) Lipases: interfacial enzymes with attractive applications. Angew. Chem. Int. Ed. Engl. 37, 1608–1633.
[2] Reetz, M.T. (2002) Lipases as pratical biocatalysts. Curr. Opin. Chem. Biol. 6, 145–150.
[3] Svendsen, A. (2000) Lipase protein engineering. Biochim. Biophys. Acta 1543 (2), 223–238.
[4] Brocca, S., Secundo, F., Ossola, M., Alberghina, L., Carrea, G. and Lotti, M. (2003) Sequence of the lid affects activity and specificity of *C. rugosa* lipase isoenzymes. Protein Sci. 12, 2312–2319.
[5] Schmitt, J., Brocca, S., Schmid, R.D. and Pleiss, J. (2002) Blocking the tunnel: engineering of *Candida rugosa* lipase mutants with short chain length specificity. Protein Eng. 15 (7), 595–601.
[6] Holmquist, M., Tessier, D.C. and Cygler, M. (1997) Identification of residues essential for differential fatty acyl specificity of *Geotrichum candidum* lipases I and II. Biochemistry 36 (48), 15019–15025.
[7] Klein, R.R, King, G., Moreau, R.A. and Haas, M.J. (1997) Altered acyl chain length specificity of *Rhizopus delemar* lipase through mutagenesis and molecular modeling. Lipids 132 (2), 123–130.
[8] Martinelle, M., Holmquist, M., Clausen, I.G., Patkar, S., Svendsen, A. and Hult, K. (1996) The role of Glu87 and Trp89 in the lid of *Humicola lanuginosa* lipase. Protein Eng. 9 (6), 519–524.
[9] Yang, J., Koga, Y., Nakano, H. and Yamane, T. (2002) Modifying the chain-length selectivity of the lipase from *Burkholderia cepacia* KWI-56 through in vitro combinatorial mutagenesis in the substrate-binding site. Protein Eng. 15 (2), 147–152.
[10] Ollis, D.L., Cheah, E., Cygler, M., Dijkstra, B., Frolow, F., Franken, S.M., Harel, M., Remington, S.J., Silman, I., Schrag, J., Sussman, J.L., Verschueren, K.H.G. and Goldman, A. (1992) The alpha/beta-hydrolase fold. Protein Eng. 5, 197–211.
[11] Pleiss, J., Fischer, M. and Schmid, R.D. (1998) Anatomy of lipase binding site: the scissile fatty acid binding site. Chem. Phys. Lipids 93, 67–70.
[12] Holmquist, M., Martinelle, M., Clausen, I.B., Patkar, S., Svendsen, A. and Hult, K. (1994) Trp89 in the lid of *Humicola lanuginosa* lipase is important for efficient hydrolysis of tributyrin. Lipids 29 (9), 599–603.
[13] Dugi, K.A., Dichek, H.L. and Santamarina-Fojo, S. (1995) Human hepatic and lipoprotein lipase: the loop covering the catalytic site mediates lipase substrate specificity. J. Biol. Chem. 270 (43), 25396–25401.
[14] Verger, R. (1997) 'Interfacial activation' of lipases: facts and artifacts. Trends Biotechnol. 15, 32–38.
[15] Alquati, C., De Gioia, L., Santarossa, G., Alberghina, L., Fantucci, P. and Lotti, M. (2002) The cold-active lipase of *Pseudomonas fragi*. Heterologous expression, biochemical characterization and molecular modeling. Eur. J. Biochem. 269, 3321–3328.
[16] Schrag, J.D., Li, Y., Cygler, M., Lang, D., Burgdorf, T., Hecht, H.-J., Schmid, R., Schomburg, D., Rydel, T.J., Oliver, J.D., Strickland, L.C., Dunaway, C.M., Larson, S.B., Day, J. and McPherson, A. (1997) The open conformation of a *Pseudomonas* lipase. Structure 5, 187–202.
[17] Nardini, M., Lang, D.A., Liebeton, K., Jaeger, K.E. and Dijkstra, B.M. (2000) Crystal structure of *Pseudomonas aeruginosa* lipase in the open conformation – The prototype for family I.1 of bacterial lipases. J. Biol. Chem. 275, 31219–31225.
[18] Lang, D.A., Hofmann, B., Haalck, L., Hecht, H.-J., Spener, F., Schmid, R.D. and Schomburg, D. (1996) Crystal structure of a bacterial lipase from *Chromobacterium viscosum* ATCC 6918 refined at 1.6 angstrom resolution. J. Mol. Biol 259, 704–717.
[19] Jaeger, K.E., Ransac, S., Dijkstra, B.W., Colson, C., van Heuvel, M. and Misset, O. (1994) Bacterial lipases. FEMS Microbiol. Rev. 15, 29–63.
[20] Bornscheuer, U., Reif, O.-W., Lausch, R., Freitag, R., Scheper, T., Kolisis, F.N. and Menge, U. (1994) Lipase of *Pseudomonas cepacia* for biotechnological purposes – Purification, crystallization and characterization. Biochim. Biophys. Acta 1201, 55–60.
[21] Mannesse, M.L.M., Boots, J.W.P., Dijkman, R., Slotboom, A.J., Vanderhijden, H.T.W.V., Egmond, M.R., Verheij, H.M. and de Haas, G.H. (1995) Phosphonate analogs of triacylglycerols are potent inhibitors of lipase. Biochim. Biophys. Acta 1259, 56–64.
[22] Lang, D.A. and Dijkstra, B.W. (1998) Structural investigations of regio- and enantioselectivity of lipases. Chem. Phys. Lipids 93, 115–122.
[23] Sambrook, K.J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, second ed, Cold Spring Harbor Laboratory Press, Plainview, New York.
[24] Bradford, M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.
[25] Humphrey, W., Dalke, A. and Schulten, K. (1996) VMD – Visual Molecular Dynamics. J. Mol. Graphics 14, 33–38.
[26] Joerger, R.D. and Hass, M.J. (1994) Alteration of chain length selectivity of a *Rhizopus delemar* lipase through site-directed mutagenesis. Lipids 29, 377–384.
[27] Gonzalez-Navarro, H., Bano, M.C. and Abad, C. (2001) The closed/open model for lipase activation. Addressing intermediate active forms of fungal enzymes by trapping of conformers in water-restricted environments. Biochemistry 40 (10), 3174–3183.